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Review

Human and viral nucleoside/nucleotide kinases involved in antiviral drug activation: Structural and catalytic properties

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ABSTRACT

Antiviral nucleoside and nucleotide analogs, essential for the treatment of viral infections in the absence of efficient vaccines, are prodrug forms of the active compounds that target the viral DNA polymerase or reverse transcriptase. The activation process requires several successive phosphorylation steps catalyzed by different kinases, which are present in the host cell or encoded by some of the viruses. These activation reactions often are rate-limiting steps and are thus open to improvement. We review here the structural and enzymatic properties of the enzymes that carry out the activation of analogs used in therapy against human immunodeficiency virus and against DNA viruses such as hepatitis B, herpes and poxviruses. Four major classes of drugs are considered: thymidine analogs, non-natural L-nucleosides, acyclic nucleoside analogs and acyclic nucleoside phosphonate analogs. Their efficiency as drugs depends both on the low specificity of the viral polymerase that allows their incorporation into DNA, but also on the ability of human/viral kinases to provide the activated triphosphate active forms at a high concentration at the right place. Two distinct modes of action are considered, depending on the origin of the kinase (human or viral). If the human kinases are house-keeping enzymes that belong to the metabolic salvage pathway, herpes and poxviruses encode for related enzymes. The structures, substrate specificities and catalytic properties of each of these kinases are discussed in relation to drug activation.

Contents

1. Introduction 102
2. General properties of the kinases and the chemistry of phosphate transfer 102
3. The first phosphorylation step 105
   3.1. Human nucleoside kinases from the salvage pathway 105
   3.2. Viral nucleoside kinases and alternate processes 108

Abbreviations: ANP, acyclic nucleoside phosphonates; Acyclovir, 9-(2-hydroxyethoxymethyl)guanine; Adefovir, PMEA, 9-2-(phosphonomethoxy)ethyladenine; AZT, (2′,3′-dideoxy-3′-azidothymidine); BVdU, brivudin, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; CE, catalytic efficiency; Cidofovir, (*S*)-1-(3-hydroxy-2-phosphonylmethoxy-propyl) cytosine; CMV, cytomegalovirus; ddT, 2′,3′-dideoxy-thymidine; d4T, Stavudine, 2′,3′-didehydro-2′,3′-dideoxy-thymidine; Ganciclovir, 9-(1,3-dihydroxy-2-propoxymethyl)guanine; HBV, hepatitis B virus; HIV, human immunodeficiency virus; HSV, herpes simplex virus; IdU, Idoxuridine, (5-Iodo-2′-deoxyuridine); Lamivudine, 3TC, (β-L-2′,3′-dideoxy-3′-thiacytidine); RT, reverse transcriptase; Telbivudine, L-dT, (β-L-2′-deoxythymidine); Tenofovir, (*R*)PMPA, (*R*)-9-(phosphonomethoxy)propyl-adenine; Torcitabine, L-dC, (L-2′-deoxycytidine); Vacc, vaccinia virus; VZV, varicella zoster virus.

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4. The second phosphorylation step 109
4.1. Human nucleoside monophosphate kinases from the salvage pathway: general properties 109
4.2. Human UMP-CMP kinase 110
4.3. Human adenylate kinases 110
4.4. Human guanylate kinases 111
4.5. Human thymidylate kinase 111
4.6. Poxvirus thymidylate kinase 112
5. Third phosphorylation step 112
5.1. Nucleoside diphosphate kinase 112
5.2. Phosphoglycerate kinase and other human kinases 115
6. Improving the kinases efficiency and changing their specificity 115
7. Conclusion 116
Acknowledgements 116
References 116

1. Introduction

Recent reviews have been dedicated to the discovery and development of antiviral drugs, their pharmacokinetics and their therapeutic use (De Clercq, 2004; Biron, 2006; Naesens and De Clercq, 2001; Cheng, 2001; Coen and Schaffer, 2003; Smee and Sidwell, 2003; De Clercq and Neyts, 2004; Mathé and Gosselin, 2006). Among these drugs, a number are nucleoside analogs that were originally developed in the frame of basic cancer research programs studying purine and pyrimidine metabolic pathways (Biron, 2006). 5-Fluoro-uracyl (5-FU), the 5-fluoro-uridine precursor basis, was used for the first time in clinics as an antineoplasic agent (Heidelberger et al., 1957). In 1963, William Prusoff introduced the isosteric 2′-deoxythymidine analog 5-iodo-2′deoxyuridine (IdU) for treatment of Herpes simplex virus (HSV) keratitis and of vaccinia virus (Vacc) infections (Prusoff, 1963; Prusoff et al., 1963; Tamm and Eggers, 1963). Twenty years later, the emergence of the human immunodeficiency virus (HIV) and the resulting acquired immunodeficiency syndrome (AIDS) pandemic greatly stimulated that field of study. After the approval of 2′,3′-dideoxy-3′-azidothymidine (AZT) against AIDS in 1987, a series of new nucleoside analogs were developed as antiviral agents. They all target the viral DNA polymerase or reverse transcriptase of HIV (HIV-1 RT), and must be processed to their triphosphate form, which competes with the natural substrates for incorporation in newly synthesized DNA. The triphosphorylated derivatives cannot themselves be used as drugs, because, due to their polarity, they cannot cross the cell membranes. Thus, the activation relies on the anabolism of the nucleoside parents (which can be seen as prodrugs of active nucleoside triphosphates), which is generally found to be inefficient when the cellular amount of phosphorylated derivatives is measured, with severe consequences on the therapeutic value of the analogs.

The process from nucleosides to nucleoside triphosphates involves successive phosphorylations often catalyzed by several cellular enzymes, in particular the kinases of the nucleoside salvage pathway, but also by viral kinases. The involvement of salvage pathway kinases (Fig. 1) has been demonstrated in several studies. The overexpression of thymidylate kinase in an inducible cell line yields higher amounts of the di- and tri-phosphate forms of two thymidine analogs, e.g. the L-FMAU (1-(2′-deoxy-2′-fluoro-β-L-*arabino*furanosyl)-5-methyluracil, Clevudine™), an efficient inhibitor of the replication of hepatitis B virus (HBV) and the d4T (2′,3′-didehydro,-2′,3′-deoxythymidine, Stavudine™) (Fig. 2), a major anti-HIV drug (Hu et al., 2005). For deoxycytidine kinase, a partial deletion of the gene in human cells is at the origin of the resistance to the anticancer agent Gemcitabine™ (2′-deoxy, 2′,2′difluorocytidine) (Galmarini et al., 2004; Jordheim and Dumontet, 2007). In addition to the cellular kinases, the nucleoside and nucleotide kinases coded by DNA viruses such as herpes viruses: Herpes simplex virus (HSV), varicella zoster virus (VZV), cytomegalovirus (CMV), and poxviruses (vaccinia, variola), are involved in the phosphorylation process, opening the way to the development of compounds that are selectively recognized as substrates by the viral enzyme but not by its human counterparts (Fyfe et al., 1978). Thus antiviral nucleoside analogs rely on two distinct modes of action, according to the specificities of viral and human related kinases. Fig. 3 illustrates this point for the two major drugs AZT and ganciclovir.

Given the abundant literature on the antiviral analogs, why provide a new review on their phosphorylation? The reason is that this process determines their efficiency as drugs: a phosphorylation step is almost always a rate-limiting step in the overall activation pathway. As the first phosphorylation catalyzed by nucleoside kinases is often said to be a rate limiting, this statement has to be reexamined in the light of studies on the nucleotide kinases that carry out the second and third steps, and may be less efficient than the nucleoside kinases on some of the analogs. Other relevant steps, such as the cell transport of nucleosides (Smith et al., 2007a; Young et al., 2008), the role of nucleotidases (Mazzon et al., 2003) and of degradation enzymes such as UDP-glucuronidase (Barbier et al., 2000; Belanger et al., 2009) are not within the scope of this review. Other metabolic enzymes not considered here are implicated in the toxicity of antiviral nucleoside analogs, for example thymidylate synthase (Balzarini et al., 1993).

We will focus on the structural and kinetic properties of the salvage pathway enzymes and underline the elements that govern their efficiency in the activation of nucleoside analogs. Four types of FDA-approved antiviral analogs licensed for clinical use are considered (Fig. 2, Table 1): (A) thymidine analogs such as AZT, d4T, BVdU; (B) acyclic nucleoside analogs such as acyclovir, ganciclovir; (C) L-nucleosides such as 3TC, L-dT, L-dC; (D) acyclic nucleotide analogs such as PMEA, PMPA, cidofovir.

2. General properties of the kinases and the chemistry of phosphate transfer

The kinases of the salvage pathway and those coded by viral genomes are all small proteins (17–45 kDa). A majority is monomeric, but dimers, tetramers and hexamers also exist. They do not behave as allosteric enzymes, with some exceptions such as deoxycytidine kinase (dCK), hTK2 and the ATP-regulated human thymidine kinase 1 (hTK1) displaying a negative cooperativity towards their substrate 2′-deoxycytidine or 2′-deoxythymidine (Munch-Petersen et al., 1991). Moreover dNKs, the enzymes catalyzing the addition of the first phosphate group, are inhibited by the end product dNTP, binding as a bi-substrate as explained below.

Kinases catalyze the transfer of a phosphate group from a donor, usually the γ-phosphate of ATP, to an acceptor, either the 5′-OH group of nucleoside, or the α- or β-phosphate group of a nucleotide. The donor and acceptor bind at two distinct sites, usually in a non-ordered manner, although binding of the first substrate may facilitate or inhibit binding the second one. Two exceptions are nucleoside diphosphate kinase (NDPK), which has a single site for

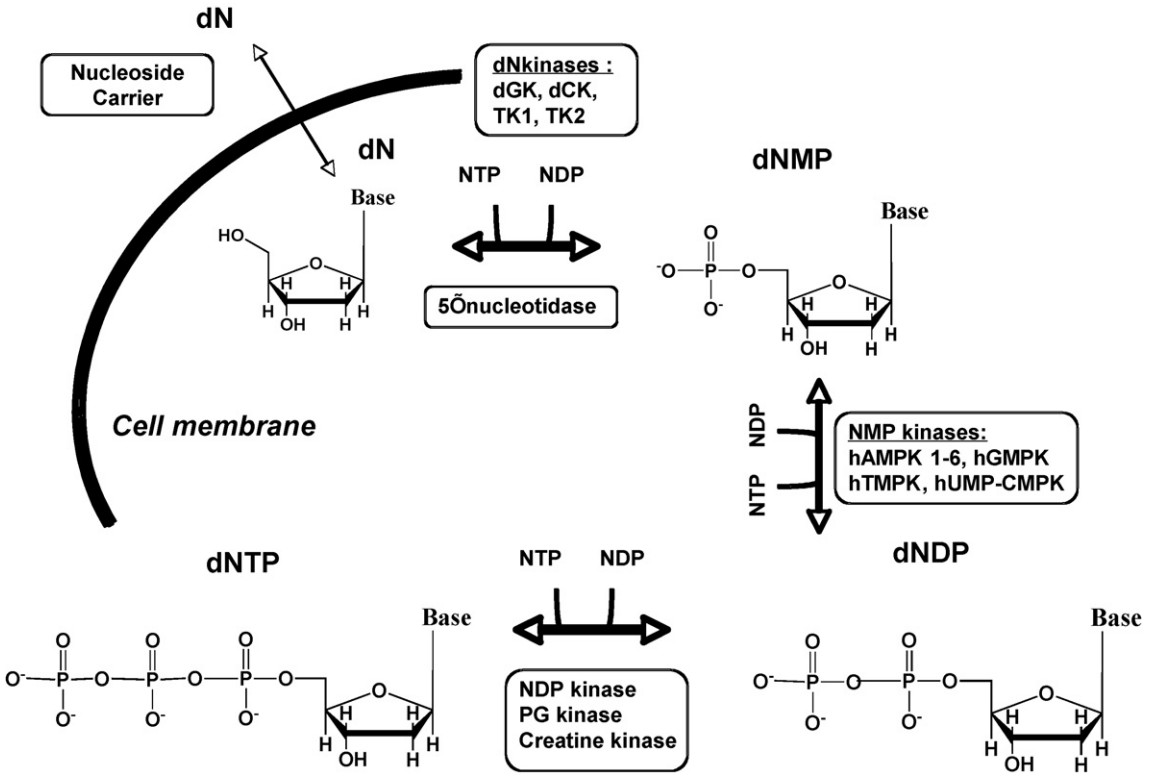

Fig. 1. Salvage pathway for natural nucleosides in human. The stepwise phosphorylation of a nucleoside into a nucleoside triphosphate implicates: (Step 1, deoxynucleoside kinases) deoxyguanosine kinase, deoxycytidine kinase, thymidine kinases 1 and 2; (Step 2, nucleoside monophosphate kinases) adenylate kinases 1 to 6, guanylate kinase, thymidylate kinase, uridylate-cytidylate kinase; (Step 3) nucleoside diphosphate kinase, phosphoglycerate kinase, creatine kinase.

both substrates, and the thymidine kinase of Herpes simplex virus type 1 (HSV1-TK), which binds thymidine before ATP (Perozzo et al., 2000). The phosphate transfer reaction occurs when the nucleophilic oxygen of the sugar 5′-OH or the acceptor phosphate attacks the γ-phosphorus atom of the donor. The process takes place 'in-line', and this requires that the donor and the acceptor are correctly positioned, but also may involve conformation changes in the enzyme, such as 'LID closing' or hinge bending domain movements (Vonrheim et al., 1995; Aden and Wolf-Watz, 2007). During the reaction, the γ-phosphorus changes from a tetrahedral to a planar trigonal configuration (Benkovic and Schray, 1968). This can be mimicked by aluminum fluoride (AlF_3) (Fig. 4B). Several X-ray structures show AlF_3 positioning itself between the β-phosphate of ADP and the acceptor substrate at the active site of a signaling

Table 1
Classes of antiviral nucleoside analogs.

Abbreviation	Generic name	1st FDA approval date[a]	Virus
A. Thymidine analogs			
AZT	Zidovudine	March 1987	HIV-1
d4T	Stavudine	June 1994	HIV-1
IdU	Idoxuridine	<January 1982	Herpes, CMV
BVdU	Brivudin	–	Herpes
B. Acyclic nucleosides			
ACV	Acyclovir	March 1982	Herpes, CMV
GCV	Ganciclovir	June 2003	Herpes, CMV
C. L-Nucleosides			
L-3TC	Lamivudine	November 1985	HIV, HBV
L-dT	Telbivudine	Oct 2006	HBV
Val-L-dC	Valorcitabine (monoVal)	–	HBV
D. Acyclic nucleoside phosphonates			
PMEA	Adefovir (dipivoxil)	September 2002	HBV
PMPA	Tenofovir (disoproxil fumarate)	October 2001, August 2004	HIV HIV, HBV
CDV	Cidofovir	June 1996	AIDS associated-CMV retinitis

The four classes of antiviral nucleoside and nucleotide analogs mentioned in this review are used in clinics. Chemical names are given in Fig. 2. Several molecules (L-dC, PMEA, PMPA) are given to patients as "pronucleotides" bearing chemical modifications that increase their biodisponibility, stability and delivery into cells. The date of approval by the Food and Drug Administration and the virus therapy concerned are cited. HIV-1, human immunodeficiency virus type 1; Herpes: Herpes simplex virus; CMV: cytomegalovirus; HBV: hepatitis B virus; AIDS acquired immunodeficient symdrome.

[a] From Orange book in http://www.fda.gov/.

Table 2
Major human and virus deoxyribonucleoside kinases (dNK).

Abbreviation	Name	Natural substrates	Sub-cellular location	length	Molecular weight (Da)	Oligomeric state	PDB entries with antiviral analog	Ref.
hdCK	Human deoxycytidine kinase EC 2.7.1.74	dC, dG, dA	Cytosol	260	30,519	Dimer	2noa, 2no7, 3exk	Sabini et al. (2003, 2007)
hdGK	Human deoxyguanosine kinase EC 2.7.1.113	dG, dA,	Mitochondria	277	32,054	Dimer	1jak, 2ocp	Johansson et al. (2001)
hTK1	Human thymidine kinase 1 EC 2.7.1.21	dT, dU	Cytosol	234	25,397	Dimer and tetramer	1xbt	Welin et al. (2004) and Birringer et al. (2005)
hTK2	Human thymidine kinase 2 EC 2.7.1.21	dT, dU, dC	Mitochondria	266	31,142	Dimer	Model based on 1ot3	Barroso et al. (2003)
HSV1-TK	Herpes simple virus-1 thymidine kinase EC 2.7.1.21	dT, dC, dTMP	–	376	40,913	Dimer	1ki2, 1ki7, 1ki5, 1ki8	Wild et al. (1995) and Brown et al. (1995)
VZV-TK	Varicella zoster virus thymidine kinase (HHV3) EC 2.7.1.21	dT, dC, dTMP	–	341	37,843	Dimer	1osn	Bird et al. (2003)
Vacc-TK	Vaccinia virus thymidine kinase (strain Copenhagen) EC2.7.1.21	dT, dC	–	176	20,102	Tetramer	2j87	El Omari et al. (2006)

The data on cellular localization, number of amino-acids and molecular weight are from Swiss Prot data bank (http://www.expasy.ch/sprot).
PDB entry codes refer to X-ray structures deposited in the Protein Data Bank (PDB, http://www.rcsb.org/pdb) of kinases complexed with antiviral nucleoside analogs.

kinase such as Ras (Scheffzek et al., 1997), and some nucleotide kinases (Xu et al., 1997b; Schlichting and Reinstein, 1999). In the planar trigonal intermediate, the phosphorus may in principle be linked to both the leaving and the attacking group, or to neither (Fig. 4A). The reaction mechanism is said to be associative in the first case, dissociative in the second. Whereas the non-enzymatic reaction is usually dissociative (Admiraal et al., 1999), the mechanism of the kinase-catalyzed transfer may be anywhere in between the two extremes (Hutter and Helms, 2000). The negative electric charge of the phosphate group, which can be minus one to minus three depending on the mechanism, is neutralized by the divalent metal (usually Mg^{2+}), which all kinases require for activity, and by the charged groups of Lys or Arg residues in the active site. This is common to all kinases for activity. The reactions catalyzed by nucleotide kinases are reversible and proceed near thermodynamic equilibrium (Yan and Tsai, 1999; Lascu and Gonin, 2000). The ones catalyzed by nucleoside kinases are not reversible, and the dephosphorylation of their product requires 5′-nucleotidases, not discussed here (Mazzon et al., 2003).

The tables reported in this review describe the properties of the major human enzymes involved in each phosphorylation step: deoxynucleoside kinases (dNK), nucleoside monophosphate kinases (NMPK), and nucleoside diphosphate kinases (NDPK). Tables 2, 4 and 6 account for their specificity for natural substrates, subcellular location, amino acid number, molecular weight, quaternary structure, and PDB entry codes that point to X-ray struc-

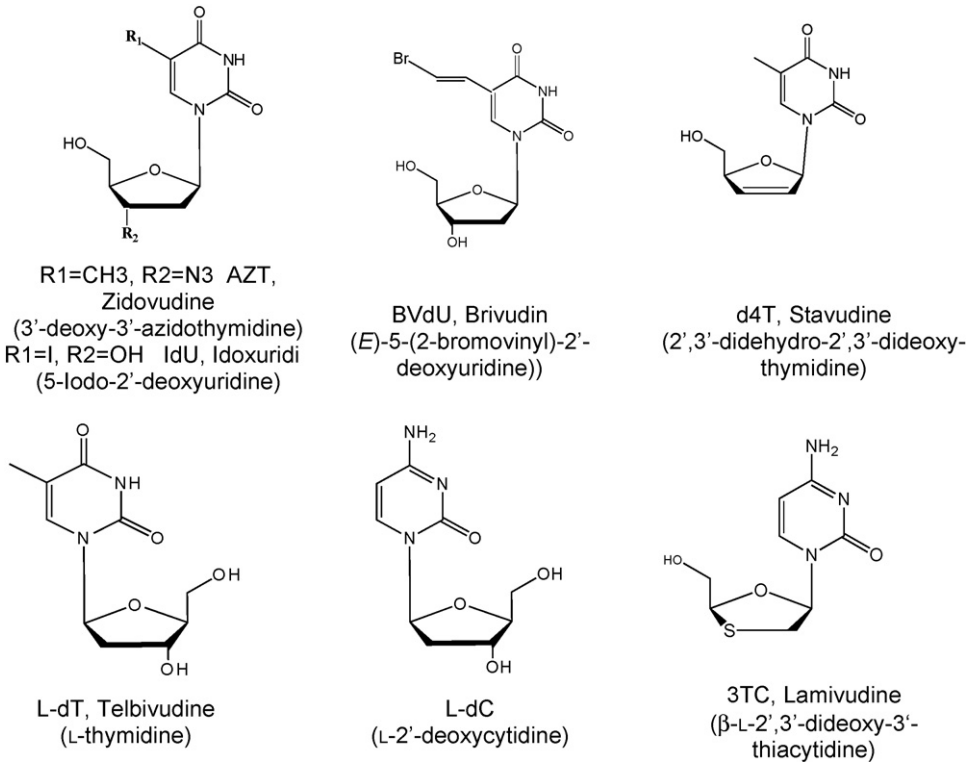

Fig. 2. Nucleoside analogs in antiviral therapies. Nomenclature and chemical formula of nucleoside analogs and acyclic nucleoside analogs and acyclic nucleoside phosphonates (ANP).

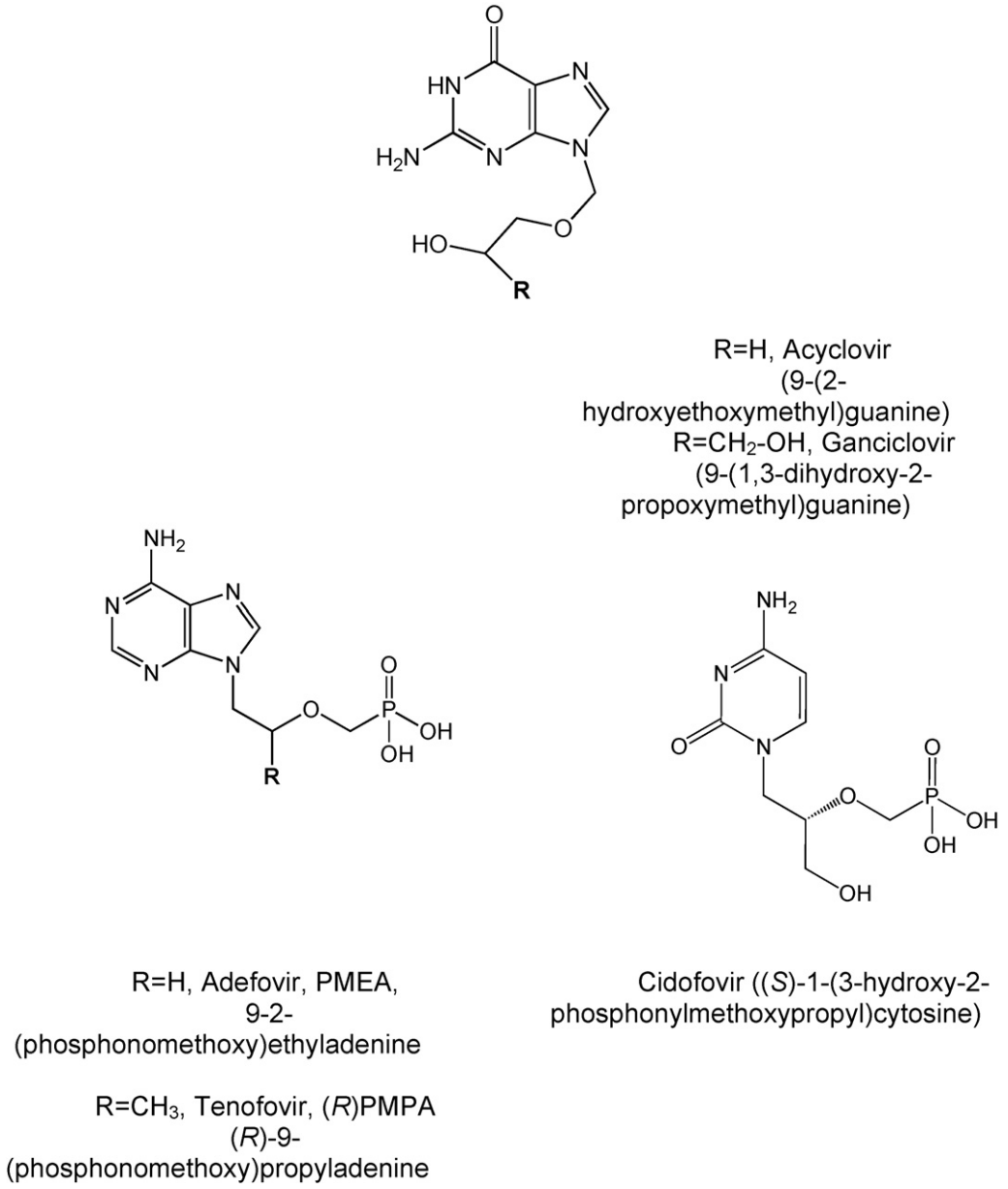

Fig. 2. (*continued*)

tures deposited in the Protein Data Bank (Berman et al., 2000). Tables 3, 5 and 7 characterize the substrate properties of antiviral nucleoside and nucleotide analogs, in the form of the catalytic efficiency (k_{cat}/K_M) measured in kinetic experiments; individual values of k_{cat} and K_M can be found in the references provided with the tables. The tables compile published enzymatic data, which sometimes revealed large discrepancies that may be due either to the drawbacks of each type of assay (most often radiometric or spectrophotometric), or to the state of the protein, its stability and mode of conservation. In addition, some data in the literature were often incomplete, or they lacked important information such as the enzyme concentration, and had to be excluded.

3. The first phosphorylation step

3.1. Human nucleoside kinases from the salvage pathway

We describe herein major features of these enzymes, and refer the reader for additional information to several excellent reviews (Arner and Eriksson, 1995; Eriksson et al., 2002; Van Rompay et al., 2000; Van Rompay et al., 2003). In human cells, the phosphorylation of nucleosides at 5′ position is catalyzed by two cytosolic enzymes, thymidine kinase 1 (hTK1) and deoxycytidine kinase (dCK), and by two mitochondrial enzymes, thymidine kinase 2 (hTK2) and deoxyguanosine kinase (dGK). The four enzymes have distinct but overlapping specificities (Table 2).

Based on their amino acid sequences, the deoxynucleoside kinases have been divided into two families. The first dNKs family contains hdCK, hdGK and the mitochondrial hTK (so-called hTK2), which have more than 40% sequence identity. It also contains the *Drosophila melanogaster* deoxynucleoside kinase (Dm-dNK), which accepts all nucleosides as substrates, and the viral HSV-1TK, two proteins that are more divergent in their sequence but have a similar 3D-structure (Welin et al., 2004). The enzymes of this family are homodimers, and they resemble thymidylate kinase in their structure, but the substrate cleft is less pronounced in hdGK and Dm-dNK (Johansson et al., 2001). The other family includes cytosolic hTK1 and vaccinia virus TK, which have been classified as type

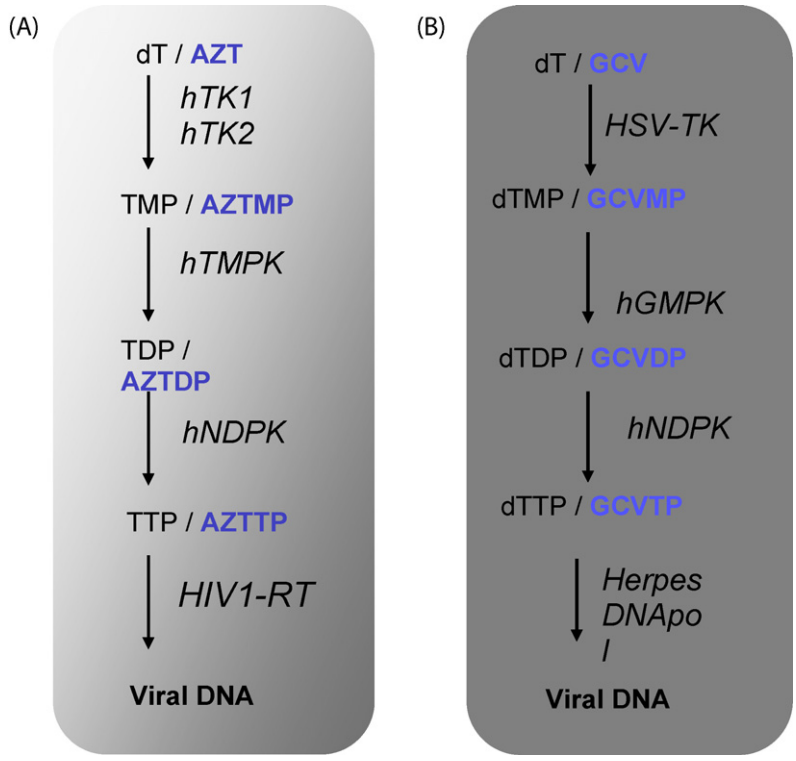

Fig. 3. Activation mechanism of nucleoside analogs in HIV-1 infected cells treated with AZT, and in HSV-1 infected cells treated with ganciclovir (GCV). The human enzymes of the salvage pathway and the viral thymidine kinase contribute to the activation of the analogs. *HIV-1 RT*: HIV-1 reverse transcriptase, *Herpes DNApol*: Herpes DNA polymerase.

II thymidine kinases, in a rather confusing manner. These TKs are homotetramers (Table 2).

The PDB contains many entries for human dNKs, but all, except a bacterial TK are for liganded forms of the enzymes. The structure of dCK, shown in Fig. 5A, is representative of the family. The monomer is an α/β domain built on a five-stranded parallel β-sheet surrounded by ten α-helices; the dimer interface is a four-helix bundle composed of helices α4 and α7 from each monomer (Sabini et al., 2003). Three conserved sequence motifs play a specific role in dNKs: the P-loop motif that binds the phosphates of ATP, the Glu–Arg–Ser sequence where the glutamate contributes to Mg^{2+} binding and the arginine has a catalytic role, and the so-called "LID" domain that contains three conserved arginines. The LID domain closes onto the bound phosphoryl donor and supplies residues that participate in ATP binding and catalysis (Johansson et al., 2001).

TK1 has a quite different structure, shown in Fig. 5B with bound dTTP, a bi-substrate inhibitor (see below). The protein is a tetramer, and the subunit contains both an α/β domain and a structural zinc domain. A 'lasso loop' binds thymidine, and ATP binding site induces a significant quaternary structure reorganization (Welin et al., 2004; Segura-Pena et al., 2007).

Table 3
Catalytic properties of human dNKs with natural substrates and antiviral analogs.

Substrate	hTK1 k_{cat}/K_M (M^{-1} s^{-1})	hdCK k_{cat}/K_M (M^{-1} s^{-1})	hTK2 k_{cat}/K_M (M^{-1} s^{-1})	hdGK k_{cat}/K_M (M^{-1} s^{-1})	HSV1-TK → monoP k_{cat}/K_M (M^{-1} s^{-1})	Vacc-TK k_{cat}/K_M (M^{-1} s^{-1})
dT	*8×10^6* [a,i]	2×10^2 [i]	*9×10^5* [a,i]	nd [i]	*1.2×10^6* [c,f]	8×10^3 [r,s,t]
AZT	3×10^6 [a,b,i]	nd	8×10^4 [a,i]	nd [i]	1.1×10^4 [f,n]	+ [l]
d4T	12 [a,d]	nd	Weak	nd	39 [d]	
IdU	8×10^6 [a,t]				1.3×10^6 [p]	9×10^3 [t]
BVdU	nd	nd	2×10^5 [h,m,k]	nd	10^6 [k,p]	–
L-dT	Weak [g]	7×10^4 [g]	2×10^5 [h,m]	nd	10^6 [l]	–
dC	nd	*2×10^5* [g,i]	8×10^5 [h]	1×10^3 [i]	$>1.2 \times 10^6$ [p]	10^2 [r]
L-dC	nd	7×10^4 [o]	6×10^5 [h]	2.9×10^3 [h]	$<10^4$ [l]	–
L-3TC	nd	1×10^5 [i]	10^2 [h]	nd	nd	–
dG	nd[h]	6×10^4 [g]	nd[h]	*6×10^4* [j,i]	5×10^4 [p]	nd[r]
Acyclovir	nd	nd [j]	nd	nd [j]	36 [c]–300 [n,q]	nd[l]
Ganciclovir	nd	nd	nd	5×10^2 to 10^3 [j]	2 to 6.8×10^3 [c,e,q]	nd

nd: not detectable; +, active but no measurable CE; – no available data.
Catalytic efficiencies (CE) were derived from values of the turnover number k_{cat} and Michaelis constant K_M, found in literature, based on initial rates measurements as a function of substrate concentration under steady state conditions at constant ATP concentration (usually 1–5 mM).
Values in italics are the highest ones reported for the natural substrate. When the literature reports V_m instead of k_{cat}, the CE for the antiviral analogs have been standardized to that for natural substrate. Catalytic efficiencies may also be determined at low substrate concentrations (below K_M) as the slope of the initial rate as a function of substrate concentration.
References for k_{cat} and K_M: a = (Munch-Petersen et al., 1991), b = (Furman et al., 1986), c = (Kokoris and Black, 2002), d = (Drake et al., 1997), e = (Hinds et al., 2000), f = (Pilger et al., 1999), g = (Sabini et al., 2003), h = (Wang et al., 1999), i = (Eriksson et al., 2002), j = (Herrström et al., 1998), k = (Cheng et al., 1981), l = (Spadari et al., 1992), m = (Verri et al., 1997), n = (Schelling et al., 2001), o = (Shafiee et al., 1998), p = (Fyfe et al., 1978), q = (Balzarini et al., 2002a), r = (El Omari et al., 2006), s = (Smith et al., 2007b) and t = (Prichard et al., 2007).

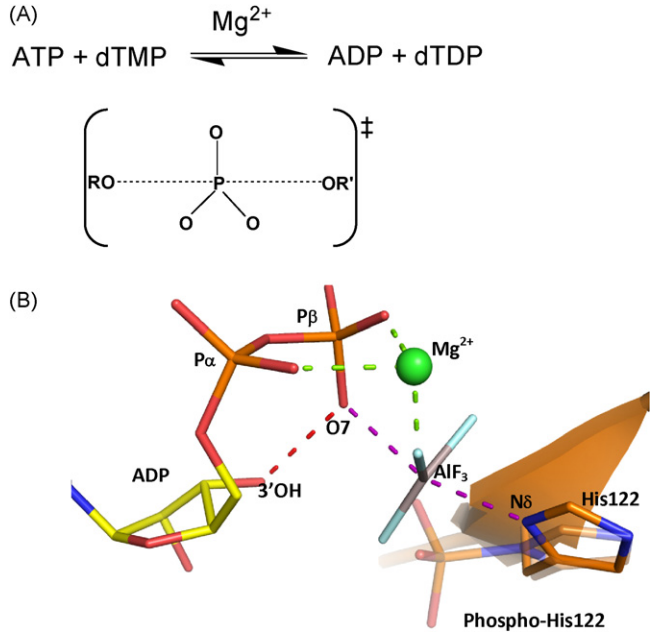

Fig. 4. A model of the in-line phosphotransfer reaction at the active site of a nucleotide kinase. (A) Scheme of the proposed transition state of the phosphoryl transfer reactions. The planar phosphoryl group (previously the γ-phosphate of ATP) is equatorially bridging (partial bonds) the phosphodonor R′ (*via* O7 of ADP moiety from ATP) and the phosphoacceptor R (dTMP) in the reaction shown above. (B) In the X-ray structure of *Dictyostelium* nucleoside diphosphate kinase in complex with ADP and aluminium fluoride (PDB entry 1kdn), AlF_3 mimics the γ-phosphate group of ATP leaving the O7 atom of the β-phosphate of ADP to make a bond with the Nδ atom of the catalytic histidine (His122 in *Dictyostelium*). The product is phosphohistidine, taken from another X-ray structure of the same enzyme (PDB entry 1nsp), and shown in superposition (transparent). In the transition state of the reaction, the γ-phosphate adopts a planar trigonal configuration with the same geometry as AlF_3, and it makes partial bonds with both O7 and Nδ. A magnesium ion (in green) ligates oxygens of the α-, β- and γ-phosphates, the latter represented in this complex by a fluorine of AlF_3, and of three water molecules (not shown). The short hydrogen bond (2.6 Å) that links O7 to the 3′-OH of the ribose, contributes to catalysis by lowering the energy of the transition state. Most of the antiviral analogs lack a 3′-OH, and this is a major cause of their poor activation by NDPK. All structural figures were generated with the program PyMOL (DeLano, 2002).

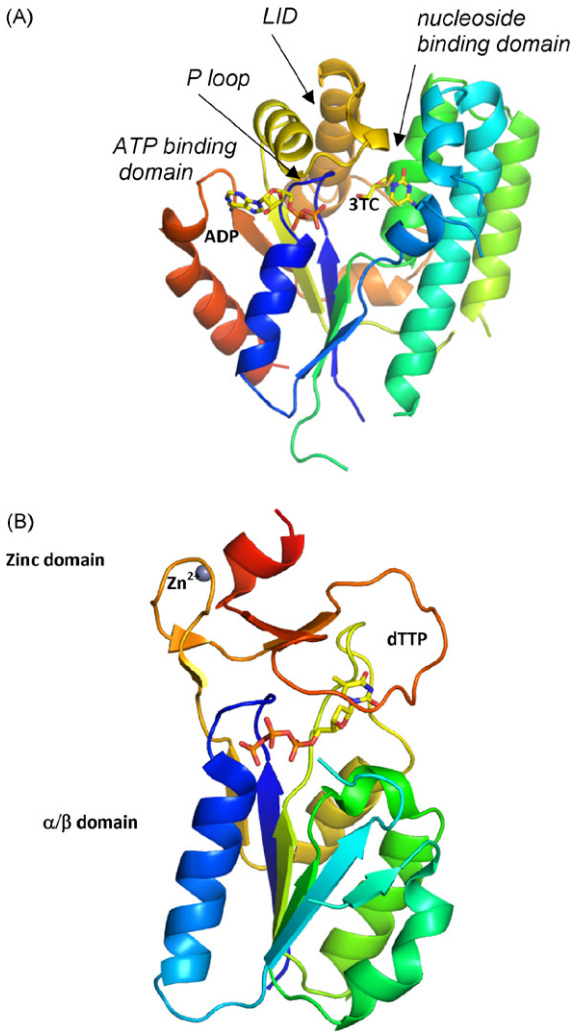

Fig. 5. The human deoxycytidine and thymidine kinases. (A) Monomer of human dCK (PDB entry 2noa) with 3TC bound at the nucleoside acceptor site on the right and ADP at the nucleotide donor site on the left of the central cleft that harbors the active site; (B) monomer of human TK1 complexed with dTTP, an inhibitor, bound at the thymidine binding site; the zinc domain on the top is unique to this kinase (PDB entry 1xbt).

The interaction of natural substrates and antiviral nucleoside analogs with dNKs has been analyzed in details (Johansson et al., 2001; Sabini et al., 2003). One common feature is the interactions of the 5′-OH of the sugar moiety with a conserved Glu–Arg pair (Glu53 and Arg128 in hdCK), involving the arginine of the conserved triad. Moreover the 3′-OH interacts with a conserved Tyr–Glu pair (Tyr86 and Glu187 in hdCK) (Sabini et al., 2003). The 5′-OH is present and the 3′-OH absent in most of the nucleoside analogs, illustrated by 3TC in the complex with hdCK (Fig. 6). The proximity of the conserved tyrosine to the 2′ position of the sugar favors deoxynucleosides over ribonucleosides (Sabini et al., 2003). In HSV-1 TK, a glutamate at the active site (Glu83, Fig. 7B) is in position to act as a base that deprotonates the 5′-OH making it a better nucleophile, whereas the positive charges of nearby Arg residues may facilitate the nucleophilic attack and subsequent transfer of the γ-phosphate (Fig. 7B) (Wild et al., 1997). As the Glu–Arg pair is conserved, this mechanism is likely applicable to all dNKs.

Table 3 indicates that the enzymes of the dNK family are relatively poor catalysts (Eriksson et al., 2002). For example, the catalytic efficiency of hdCK on its natural substrate dC is only $2 \times 10^5\ M^{-1}\ s^{-1}$, with a low K_M = 0.6–1 μM, but a turnover rate less than $1\ s^{-1}$ (Eriksson et al., 2002). Human dCK also processes dA and dG with a lower catalytic efficiency (Eriksson et al., 2002). HdCK does not obey Michaelis–Menten kinetics, but shows bimodal kinetics (Turk et al., 1999). hTK1 is much more active, with a cat-

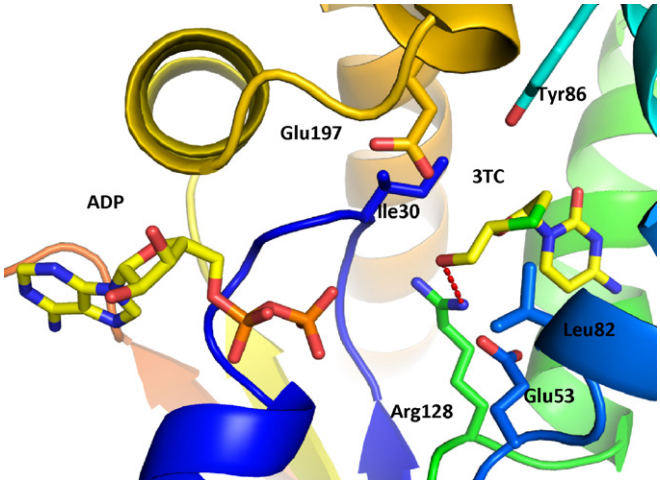

Fig. 6. 3TC at the active site of human deoxycytidine kinase. A close-up view of the ternary complex of Fig. 5A showing the bound ADP and 3TC molecules and residues that interact with the acceptor substrate (PDB entry 2noa). The red dotted line is the hydrogen bond between the 5′-OH and Arg128.

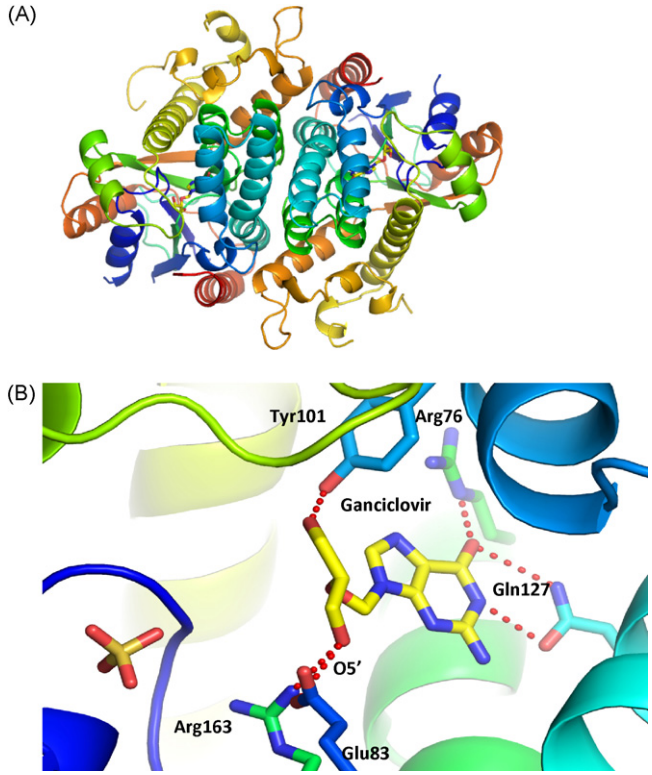

Fig. 7. Herpes simplex virus type 1 thymidine kinase. (A) HSV-1 TK dimer (PDB entry 1ki2) belongs to dNK family. (B) Ganciclovir at the nucleoside binding site of HSV1-TK (pdb id 1ki2). Hydrogen bonds are red dotted lines.

alytic efficiency that reaches $8 \times 10^6\ M^{-1}\ s^{-1}$ when fully activated. Its activity is regulated by ATP, in relation with its tetramer/dimer oligomerization (Munch-Petersen et al., 1991; Welin et al., 2004).

The end product dNTPs are dNKs competitive inhibitors, binding as bi-substrates at the enzyme active site with the base at the acceptor site and the triphosphate occupying both acceptor and donor phosphate sites. Several dNKs have been structurally solved as complexes with their respective dNTP (Johansson et al., 2001; Welin et al., 2004). Another common property of the dNKs family is their low enantioselectivity (Wang et al., 1999). The L-nucleoside analogues 3TC (anti-HIV and anti-HBV) and L-dC (anti-HBV) are almost as good substrates of hdCK as the corresponding D-enantiomers (Table 3) (Shewach et al., 1993; Sabini et al., 2007). The mitochondrial hTK2 accepts L-nucleosides such as L-dT, L-dC, L-BVdU ([*E*]-5-(2-bromovinyl)-2′deoxyuridine), L-IdU (Verri et al., 1997) as substrates, but it has very little efficiency on d4T (Munch-Petersen et al., 1991). As for the mitochondrial hdGK, it favors the D-enantiomer of dG, but it phosphorylates L-dA more efficiently than D-dA and D-dG (Gaubert et al., 1999), and accepts ganciclovir as substrate (Herrström et al., 1998).

In contrast to the mitochondrial enzymes, the cytosolic hTK1 exhibits a strict enantioselectivity and its specificity is limited to the D-enantiomer of thymidine and analogs. Human TK1 phosphorylates analogs with a modified 3′-deoxyribose sugar such as AZT and d4T, but whereas AZT is a good substrate, the activity on d4T is only 5% that on 2′-deoxythymidine (Munch-Petersen et al., 1991; Wang et al., 1999). Thymidine analogs modified at C5 of the pyrimidine are accepted when the substituent is not bulky: 5-fluoro, 5-chloro, 5-bromo, 5-iodo and 5-ethyl dU are substrates, but the anti herpetic drug [*E*]-5-(2-bromovinyl)-dU (BVDU) is not recognized. BVdU, efficient against Herpes infections, is phosphorylated by the mitochondrial hTK2 (Balzarini et al., 2000; Franzolin et al., 2006). A recent study of the incorporation of isotopically labeled dT into DNA shows that most of the mitochondrial pool of labeled dTTP is imported from the cytosol (Pontarin et al., 2003), which suggests that hTK2 contributes little to the cellular metabolism.

3.2. Viral nucleoside kinases and alternate processes

The genome of several DNA-containing viruses (herpes viruses and poxviruses) encodes a thymidine kinase. The viral TK can phosphorylate nucleoside analogs and is responsible for the virus sensitivity to these drugs (Fig. 3, Table 2). In addition to phosphorylating 2′-deoxythymidine to dTMP, the TK of herpes simplex type 1 (HSV-1) and of varicella zoster virus (VZV) have thymidylate kinase activities that convert dTMP into dTDP. They belong to the dNK family like mitochondrial hTK2, have a molecular weight around 40 kDa and are active as homodimers (Table 2). In the structure of HSV-1 TK, the first to be solved in the dNK family (Wild et al., 1995), each monomer consists of a five-stranded parallel β-sheet CORE surrounded by five CORE α-helices and five peripherical helices (Gardberg et al., 2003) (Fig. 7A). HSV-1 TK resembles *Drosophila* dNK and the NMP kinases, but in comparison to dGK, it contains an additional 45 N-terminal disordered residues, an extra domain with helices α9 and α10 inserted after helix α8, and an extra antiparallel β-strand and α-helix at the C-terminal (Wild et al., 1997; Johansson et al., 2001). The dimer interface involves helices α4, α6 and α10, and part of α2 and α12 (Wild et al., 1997). A thermodynamic study of the substrate binding to HSV-1 TK indicates that it is ordered with thymidine binding first (Perozzo et al., 2000; Wurth et al., 2001). A recent structure of the TK of equine herpes virus type 4 in complex with the bisubstrate analogs Tp4A and Tp5A gives a structural basis to the dual TK and TMPK activity (Gardberg et al., 2003). This study confirms an early prediction (Wild et al., 1997), that the dTMP phosphate group can move in the active site, undergoing a shift in position of more than 2 Å between the two complexes ADP-dTMP and ATP-dTMP.

Herpes TK shows a much broader range of activity than the host hTK1 (Table 3). HSV-1 TK can use other nucleoside triphosphates than ATP as a phosphate donor, and accepts as substrates not only the natural dT, dC and dG D-forms, but also the L-enantiomer L-dT (Spadari et al., 1992). It also phosphorylates analogs substituted at C5 of the nucleobase, including brivudin (Fyfe, 1982) (De Clercq, 2004). HSV-1 TK and HSV-2 TK also accommodate acyclic guanine nucleoside analogs such as acyclovir and ganciclovir, and add only the first phosphate group to those analogs (Fig. 3) (Vogt et al., 2000). Ganciclovir bound to HSV-1 TK is shown in Fig. 7B (Brown et al., 1995). Cells that are not infected by herpes viruses poorly activate these acyclic purine nucleoside analogs, and drug-resistant strains of herpes viruses express an inactive thymidine kinase (Larder et al., 1983). Herpes treatment has made use of the difference in substrate specificity between viral and human TKs for many years (Naesens and De Clercq, 2001; Coen and Schaffer, 2003). As developed in paragraph 6, HSV-1 TK is used together with ganciclovir as a suicide gene in a combined gene/chemotherapy strategy to treat cancer (Balzarini et al., 1985; Moolten and Wells, 1990; Culver et al., 1992; Portsmouth et al., 2007).

Ganciclovir is also approved for treatment of cytomegalovirus (CMV) infection and CMV-related diseases in transplant recipients. An unusual protein kinase homolog encoded by the viral UL97 open reading frame is responsible for the first phosphorylation of the analog (Sullivan et al., 1992; Biron, 2006), at a low extent (Zimmermann et al., 1997), even though it does not show nucleoside kinase activity (Michel et al., 1996). The protein kinase has multiple functions in virion morphogenesis (Prichard et al., 2005) and it interacts with host tumor suppressor factors such as retinoblastoma tumor suppressor (Kamil et al., 2009). Mutations in the UL97 gene, and the natural polymorphism of that gene, lead to resistance to ganciclovir (Sanchez Puch et al., 2009).

Epstein–Barr virus (EBV) encodes a large thymidine kinase that contains a N-terminal domain (243 residues) of unknown function. A truncated protein lacking the N-ter domain and expressed as a GST fusion protein, shows TK activity with a narrower substrate specificity than HSV TK – it does not phosphorylate dC, GCV or ACV (Tung and Summers, 1994) – and a minor TMPK activity (Gustafson et al., 1998).

In poxviruses, the prototype vaccinia virus codes for a TK of smaller size (Vacc-TK) sharing an extensive identity to hTK1. Its X-ray structure in a complex with dTTP, a bi-substrate feed-back inhibitor, shows a tetramer (El Omari et al., 2006) and subunits that contain both a zinc domain and a lasso domain like hTK1 in Fig. 5B. Vaccinia TK phosphorylates AZT like hTK1, and dC marginally (Table 3).

4. The second phosphorylation step

4.1. Human nucleoside monophosphate kinases from the salvage pathway: general properties

In humans, the NMPK family (Table 4) includes four enzymes named according to their preferred substrate: (d)AMP, (d)GMP, (d)CMP/(d)UMP and dTMP. Whereas human uridylate/cytidylate kinase (UMP-CMPK) has a dual specificity, (d)CMP and (d)UMP are phosphorylated by different kinases in bacteria. Humans have six isoforms of adenylate kinase (hAMPK1–6), one guanylate kinase (hGMPK), two UMP-CMPKs (hUMP-CMPK), and one thymidylate kinase (hTMPK). In addition, a putative mitochondrial thymidylate kinase has recently been described (Chen et al., 2008). All have the same highly conserved fold (Fig. 8), though their sequences diverge: that of hUMP-CMPK is 40, 27, 21 and 20% identical to those of hAMPK1, hAMPK2, hTMPK and hGMPK, respectively (Topalis et al., 2007).

A central CORE domain made of a five-strand β-sheet surrounded by 8–9 α-helices characterizes the NMPKs architecture. The CORE includes the ATP binding P-loop and has two mobile domains inserted, one that binds the (d)NMP substrate, and the LID domain that provides the catalytic residues for the reaction (Fig. 8) (Pai et al., 1977). The (d)NMP substrate interacts with the mobile domain, and also with residues of the CORE (Alexandre et al., 2007). When the substrates bind, the NMP binding and the LID domains undergo large "hinge bending" motions that make the subunit switch from an open to a closed conformation (Vonrheim et al., 1995). These conformation changes, first described in adenylate kinase (Pai et al., 1977), are believed to occur in all NMPK. A common feature to NMPKs is their inhibition by bi-substrate inhibitors such as Ap5A, shown in Fig. 8 in a complex with hAMPK1: such an inhibitor occupies the substrate cleft with the donor and acceptor nucleosides linked by a chain made of four or five phosphate groups.

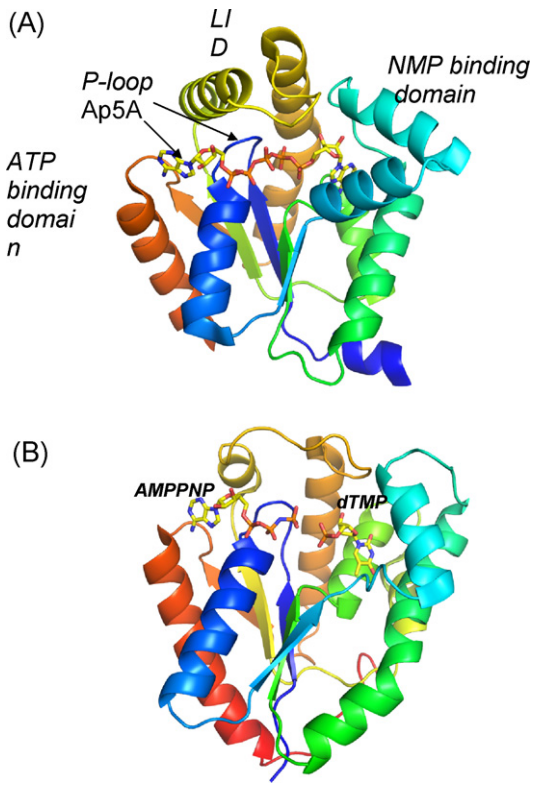

Fig. 8. The NMPK monomer fold. (A) human AMPK1 (PDB entry 1akh) complexed with Ap5A (P^1-(5′-adenosyl) P^5-(5′-adenosyl)pentaphosphate), an inhibitor that occupies both the donor nucleotide site on the left and the acceptor site on the right. (B) Human TMPK (PDB entry 1e9e) in complex with TMP and an ATP analog. UMP-CMPK and GMPK adopt the same fold as AMPK and TMPK.

Table 4
Major NMPK (step 2).

Abbreviation	Name	Natural acceptor substrates	Sub cellular location	Length	Molecular weight (Da)	Oligomeric state	PDB ID	Ref. crystal
hAMPK1	Human adenylate kinase 1 (myokinase) EC 2.7.4.3	(d)AMP	Cytosol	194	21,635	Monomer	1z83	Filippakopoulos et al. (2005)
hAMPK2	Human adenylate kinase 2 EC 2.7.4.3	(d)AMP	Mito-chondria	238	26,346	Monomer	2c9y	Bunkoczi et al., 2005
hGMPK	Human guanylate kinase (GUK1) 2.7.4.8	(d)GMP	Cytosol	197	21,594	Monomer	1lvg (mouse) 2f3t (coli)	Sekulic et al. (2002) and Hible et al. (2006)
hUMP-CMPK	Human uridylate-cytidylate kinase EC 2.7.4.14	(d)UMP (d)CMP	Cytosol	196	22,222	Monomer	1tev	Segura-Pena et al. (2004)
hTMPK	Human thymidylate kinase EC 2.7.4.9	TMP dUMP	Cytosol	212	23,833	Dimer	1nm3, 1e9b, 1e9d	Ostermann et al. (2000, 2003)
Vacc-TMPK	Vaccinia virus thymidylate kinase EC 2.7.4.9	TMP dump dGMP	–	204	23,219	Dimer	2v54	Caillat et al. (2008)

The data on cellular localization, number of amino acids and molecular weight are from SwissProt (http://www.expasy.ch/sprot).
PDB entry codes refer to X-ray structures deposited in the Protein Data Bank (PDB, http://www.rcsb.org/pdb) of kinases complexed with antiviral nucleoside analogs, except AMPK1 complexed to Ap5A, hAMPK2 to Ap4A, and hUMP-CMPK, not complexed.

4.2. Human UMP-CMP kinase

hUMP-CMPK phosphorylates dCMP more efficiently than dUMP, and is inactive on dTMP (Table 5). It has a loose enantioselectivity and accepts L-nucleotide analogs (L-dCMP, L-dUMP) as substrates, with a lower K_M than for D-dCMP and D-dUMP and with a low k_{cat} resulting in a significant catalytic efficiency (Alexandre et al., 2007). However, L-dCMP and L-dUMP are phosphorylated ten times more efficiently than L-CMP and L-UMP. L-3TCMP, the monophosphorylated form of 3TC, is a good substrate of hUMP-CMPK (Table 5) (Van Rompay et al., 1999; Liou et al., 2002; Pasti et al., 2003). The L-nucleotide analogs are not inhibitors when present in excess, as are their D-counterparts. With natural nucleotides, inhibition by excess substrate is a well-known feature of NMPKs that reflects the presence on the enzyme of two binding sites for the donor and the acceptor nucleotide, each of which may possibly accommodate the other substrate.

The structure of hUMP-CMPK has been solved as the free enzyme in open conformation (Segura-Pena et al., 2004). In the absence of structural data for hUMP-CMPK complexed with nucleotides, which may be a consequence in the crystal of an artefactual dimerization involving the LID domain of two molecules (Topalis et al., 2007), L-dCMP and D-dCMP have been docked into the CMP binding site of *Dictyostelium* UMP-CMP kinase where all the residues are conserved relative to the human enzyme (Fig. 9A) (Scheffzek et al., 1996; Schlichting and Reichstein, 1997). With both enantiomers, the cytosine ring can make the same interactions and the phosphate is in the proper place for phosphotransfer, but the sugar ring oxygen of L-dCMP makes an additional interaction with Arg96, anchoring the sugar to the NMP binding site (Alexandre et al., 2007). The oxygen in the oxathiolan ring of L-3TCMP could play the same role.

hUMP-CMPK is also responsible for the addition of the first phosphate to cidofovir, an acyclic nucleoside phosphonate with broad-spectrum anti-DNA virus activities. Cidofovir is efficient in pox infections, and approved in treatments of the retinitis due to cytomegalovirus, an opportunist infection in AIDS (Cihlar and Chen, 1996; Topalis et al., 2007). Cidofovir has a K_M similar to dCMP, but it is phosphorylated at a slow rate (less than 0.1% of dCMP rate), and the catalytic efficiency is low (Table 5). Its binding to dCMP site modeled on a complex with the *Dictyostelium* enzyme is illustrated in Fig. 9A.

4.3. Human adenylate kinases

In contrast to hUMP-CMPK, the human adenylate kinases hAMPK1 and hAMPK2 have a strict enantioselectivity, and L-dAMP is not a substrate for these enzymes (Alexandre et al., 2007). This may be a reason why no adenine derivative of the L-series has been retained as an antiviral agent. On the other hand, acyclic nucleoside phosphonates that are dAMP mimics, have been approved as prodrugs: 9-(2-(phosphonylmethoxy)ethyl) adenine (PMEA), against HBV in 2002, and (R-9-(2-(phosphonomethoxyl)propyl)adenine (PMPA) against HIV-1 in 2001 and HBV in 2004 (De Clercq and Holy, 2005). The phosphonate group replaces the first phosphate in these compounds. They bypass the initial phosphorylation catalyzed by a dNK, and rely on the cellular adenylate kinases for the second (Votruba et al., 1987; Balzarini et al., 1991; Robbins et al., 1995). Both PMEA and PMPA are slowly phosphorylated by hAMPK1 and to a larger extent, by the mitochondrial hAMPK2 (Robbins et al., 1995). With recombinant hAMPK1 and hAMPK2, the turnover is very low and the catalytic efficiency poor (Table 5) (Topalis et al., 2008). The accumulation of phosphorylated ANPs in cells may be attributed to the high cellular content of AMPK (Maughan et al., 2005) and to the long intracellular half-life of these compounds (95 h for PMPA-PP) (Balzarini et al., 2002b; Delaney

Table 5
Catalytic properties of human and viral NMPKs with some antiviral compounds compared to natural substrates.

Substrate	hTMPKk_{cat}/K_M (M^{-1} s^{-1})	hUMP-CMPKk_{cat}/K_M (M^{-1} s^{-1})	hGMPKk_{cat}/K_M (M^{-1} s^{-1})	hAMPK1k_{cat}/K_M (M^{-1} s^{-1})	hAMPK2k_{cat}/K_M M^{-1} s^{-1})	HSV_1-TKk_{cat}/K_M (M^{-1} s^{-1})	Vacc-TMPKk_{cat}/K_M (M^{-1} s^{-1})
dTMP	1.5×10^5 [a]	nd [a]	nd [a]	nd [a]	nd [a]	2.7×10^4 [j (ex)]	1×10^5 [m]
dUMP	2.8×10^4 [a]	6×10^3 [a]	nd [a]	nd [a]	nd [a]	Weak [k]	9.2×10^3 [m]
5I-dUMP	7.2×10^4 [b]	nd [b]	–	–	–		3.8×10^4 [m]
BVdUMP	nd [b]	nd [b]	nd [a]	–	–	1.5×10^3 [l,k (ex)]	4.5×10^3 [n]
AZTMP	8.3×10^2 [c]	nd [b]	–	–	–	nd [k]	1.3×10^3 [m]
d4TMP	7.5×10^3 [c,d]	nd [b]	–	–	–	nd [k]	8×10^3 [m]
L-dTMP	9×10^2 [a]	nd [b]	–	–	–		6.2×10^3 [b]
dCMP	nd [a]	7×10^4 [a]	nd [a]	nd [a]	nd [a]	nd [k]	nd [m]
L-dCMP	nd [a]	10^3 [a]	–	nd [a]	nd [a]		–
L-3TCMP	–	2.4×10^5 [e]	–	–	–		–
Cidofovir	nd [a]	60 [f]	–	nd [a]	nd [a]		nd [b]
dGMP	nd [b]	nd [a]	6×10^6 [g]	nd	nd	nd [k]	2.5×10^3 [m]
Acyclovir-MP	–	–	2×10^5 [h]	–	–	nd	–
Ganciclovir-MP	–	–	4×10^6 [h]	–	–	nd	–
dAMP	nd [a]	nd	nd [a]	1.6×10^5 [i]	5×10^5 [i]	–	nd [a]
PMEA	nd [a]	nd	–	14 [i]	2.5×10^2 [i]	–	nd [b]
PMPA	nd [a]	nd	–	75 [i]	8×10^2 [i]	–	nd [b]

nd: non-detectable; –: no available data; ex: extrapolated.
Catalytic efficiencies (CE) were derived from values of the turnover number k_{cat} and Michaelis constant K_M, found in literature, based on initial rates measurements as a function of substrate concentration under steady-state conditions at 1 mM ATP.
References for k_{cat} and K_M: a = (Alexandre et al., 2007), b = unpublished, c = (Ostermann et al., 2003), d = (Hu et al., 2005), e = (Pasti et al., 2003), f = (Topalis et al., 2007), g = (Auvynet et al., 2009), h = (Boehme, 1984), i = (Topalis et al., 2008), j = (Chen and Prusoff, 1978), k = (Balzarini et al., 2002a), l = (Fyfe, 1982), m = (Topalis et al., 2005), and n = (Caillat et al., 2008).

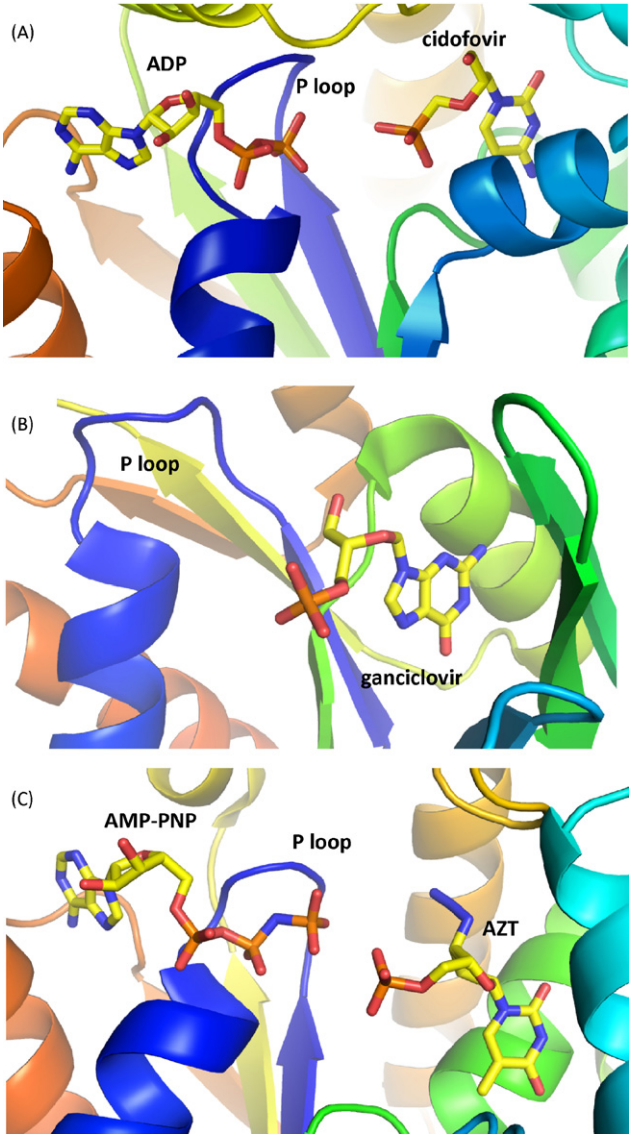

Fig. 9. Nucleotide analogs at NMPK active sites. (A) A model of cidofovir and ADP bound to hUMP-CMPK (Topalis et al., 2007), based on the *Dictyostelium* enzyme in complex with CMP and ADP (PDB entry 2ukd). (B) Ganciclovir monophosphate bound to *E. coli* GMPK active site (PDB entry 2f3t). (C) AZT monophosphate and an ATP analog bound to hTMPK (PDB entry 1e9d). In all three cases, the analog occupies the acceptor site on the right of the central cleft.

et al., 2006), that results from their insensibility to nucleotidases (Rinaldo-Matthis et al., 2004).

The 3D-structure of the adenylate kinases of many species are known, the first one having been determined by G. Schulz in the seventies (Pai et al., 1977). Those of hAMPK 1–5 have been recently deposited in PDB by the Oxford Structural Genomics Consortium, either in the open or the close conformation observed in the presence of inhibitors. However, no complex of hAMPK with antiviral analogs is available yet. When PMEA is docked in the active site of hAMPK1 (Topalis et al., 2008) and superimposed to AMP, the phosphonate group occupies the position of the α-phosphate of AMP, and it can bind to both Arg44 in the LID domain and Arg97 in the CORE domain. In contrast, the phosphono-methoxy group that replaces the deoxyribose cannot make the H-bond that natural substrate 2′OH normally gives to the main chain oxygen of Gly65, and this contributes to the loose binding of the analog.

4.4. Human guanylate kinases

Two kinds of guanylate kinases are found in human, the soluble guanylate kinase (hGMPK, also noted GUK) involved in the salvage pathway and the membrane associated guanylate kinases (MAGUK), not considered here (Li et al., 2002; Gardoni, 2008). The acyclic nucleoside analogs acyclovir and ganciclovir used against Herpes infections contain a guanine. Their first phosphorylation relies on the broadly specific Herpes thymidine kinase (Fyfe et al., 1978) (Section 3.2), but the virus kinase is not able to proceed further in the activation of theses drugs. Except for BVdUMP, which is a fairly good substrate of HSV-1 TK (Table 5), the cellular guanylate kinase is responsible for adding the second phosphate (Miller and Miller, 1980). GMPK, purified from human erythrocytes in 1984, is efficient on acyclovir monophosphate, and highly efficient on ganciclovir monophosphate, which is almost as good a substrate as dGMP, although not as good as GMP (Table 5) (Boehme, 1984).

Structural data are available for the human MAGUK, for the soluble mouse guanylate kinase, which is 88% identical to hGMPK (Sekulic et al., 2002), and for several bacterial enzymes, including a complex of *Escherichia coli* guanylate kinase with ganciclovir monophosphate (Fig. 9B). Although the *E. coli* and mammalian enzymes differ in quaternary structure (the first is a hexamer, the second a monomer), they have similar binding sites for GMP. The analog binds to the GMP binding domain like the natural substrate, but it lacks an interaction normally made by the 2′-OH (Fig. 9B) (Hible et al., 2005, 2006).

4.5. Human thymidylate kinase

Thymidylate kinase occupies a special place in the NMP kinase family. Whereas the other NMP kinases are expressed constitutively throughout the cell cycle, thymidylate kinase, like thymidine kinase, is expressed only in dividing cells, a property that explains the low toxicity of thymidine analogs (Huang et al., 1994; Eriksson et al., 2002). The structure of hTMPK is available in complex with various ligands, but not that of the free enzyme (Ostermann et al., 2000, 2003). A characteristic of the enzyme is the slow rate of phosphotransfer. The k_{cat} (near $2\,s^{-1}$) is comparable to that of the nucleoside kinase, and much less than for hUMP-CMPK and hAMPK k_{cat} with the cognate deoxynucleotides (about $100\,s^{-1}$), or adenylate kinase with AMP ($1000\,s^{-1}$). This is compensated by the low K_M of hTMPK for the acceptor substrate (0.02 mM for dTMP *vs.* 1 mM for hAMPK and dAMP), and the catalytic efficiency k_{cat}/K_M on their natural substrates, is of the order of $10^5\,M^{-1}\,s^{-1}$ range for of all the NMP kinases. Steady state kinetics analyses indicate that hTMPK has about the same K_M for the monophosphate derivatives of AZT, d4T and 5I-dUMP as for dTMP, but k_{cat} drops by a factor of 20 with d4TMP and more than 100 with AZTMP. The catalytic efficiency is low (Table 5) and hTMPK was presented for that reason as the bottleneck in the activation process of the thymidine analogs (Lavie et al., 1997a,b). Structures of hTMPK are available in complex with the monophosphate derivatives of AZT and d4T. With AZTMP (Fig. 9C), the steric hindrance created by the azido group prevents the formation of the close conformation of the P-loop, affecting catalysis (Ostermann et al., 2003). d4TMP binding in the presence of ADP or AMPPNP does not prevent closure of the P-loop, which was interpreted as the reason why this analog is a better substrate than AZTMP (Ostermann et al., 2003; Lavie and Konrad, 2004).

5-Substituted analogs such as 5-halogeno-dUMP are substrates for hTMPK, but the dTMP binding site cannot accommodate a bulky group such as bromovinyl (Topalis et al., 2005). hTMPK is enantiomer specific, and its catalytic efficiency on L-dTMP is less than 1% that on the natural substrate (Table 5), due to a turnover number k_{cat} 10 times lower and a K_M 20-fold higher. Docking of L-dTMP on the active site shows that the thymine ring can stack onto Phe72

in the L- as in the D-enantiomer and that the interactions of the phosphate group with Mg^{2+}, with Asp15 in the P-loop *via* a water molecule, and with Arg97 in the mobile part of NMP domain, are all conserved (Alexandre et al., 2007).

4.6. Poxvirus thymidylate kinase

Viral NMPKs include HSV-1 TK and VZV TK, which phosphorylates both 2′-deoxythymidine and dTMP, and the thymidylate kinase of poxvirus, which is the only virus known to code for a NMPK except for the African swine fever virus, a DNA arbovirus. The enzyme is expressed early during the infection cycle (Moss, 1990), and the gene is highly conserved in the *Orthopoxvirus* family, which includes the variola virus and all known vaccinia strains. Vaccinia virus thymidylate kinase (Vacc-TMPK) is highly similar to the human enzyme (42% identity and 64% similarity) and a homodimer like it (Topalis et al., 2005). However, the mode of subunit association and the active-site geometry are different (Caillat et al., 2008). The interface helices α3 and α6 of the two subunits, which are approximately orthogonal in Vacc-TMPK (Fig. 10A), are antiparallel in the human enzyme. In the virus protein, a canal connects the dimer interface to the thymine binding pocket. The pocket is closed by His69 in the human protein, a residue replaced in vaccinia by Asn65, the side chain of which is oriented differently. In addition, two aromatic residues (Trp116 and Phe112) are substituted by smaller residues, creating a packing defect that explains the broader substrate specificity of the viral enzyme. The dTMP binding site of Vacc-TMPK is able to accomodate substrates with larger bases, such as dGMP and BVdUMP, that are slowly phosphorylated with catalytic efficiencies about 5% that of dTMP (Fig. 10B) (Table 5) (Caillat et al., 2008). Other dTMP analogs that are substrates of hTMPK, such 5-halogeno substituted dUMP, are also substrates of Vacc-TMPK and with similar catalytic parameters (Topalis et al., 2005).

5. Third phosphorylation step

A number of enzymes from the basal metabolism can convert ADP to ATP or GDP to GTP. Some can phosphorylate other (deoxy)nucleoside diphosphates, and their possible implication in the production of the active form of the antiviral nucleosides has been studied (Table 6).

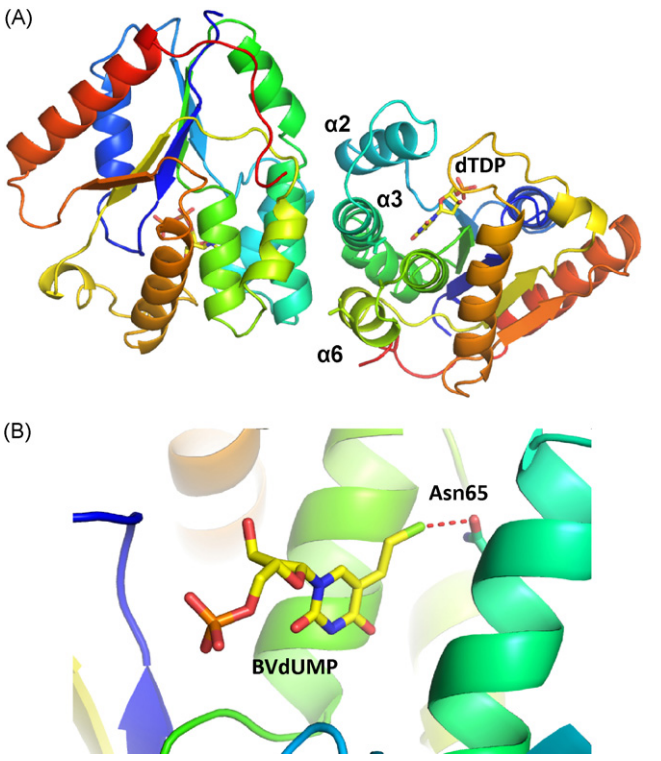

Fig. 10. Vaccinia virus thymidylate kinase. (A) The Vacc-TMPK dimer with bound TDP (PDB entry 2v54). In the human enzyme, the same helices α2, α3 and α6 form the dimer interface, but in a different orientation. (B) When BVdUMP occupies the acceptor site, an halogen bond forms between the bromine atom (green) on the modified base and Asn65 (PDB entry 2wos).

5.1. Nucleoside diphosphate kinase

Nucleoside diphosphate kinase (NDPK) catalyzes the transfer of the γ-phosphate of a (d)NTP donor nucleotide onto a (d)NDP acceptor. The donor is usually ATP or GTP, but the acceptor can be any nucleoside or deoxynucleoside diphosphate. In addition to lacking specificity towards the nature of the base or the sugar, NDPK has an unusual mechanism. Whereas most kinases directly transfer

Table 6
NDP kinase and other kinases involved in step 3.

Abbreviation	Name	Natural acceptor subtrates	Sub-cellular location	Length	Molecular weight (Da)	Oligomer	PDB ID	Ref.
NDPK-A, Nm23-H1	NDP kinase-A EC 2.7 4 6	(d)NDP	Cytosol, nucleus	152	17,149	Hexamer	2hvd (+ADP)	Chen et al. (2003)
NDPK-B, Nm23-H2	NDP kinase-B EC 2.7 4 6 Hybrids: *AxBy* ($x+y=6$)	(d)NDP	Nucleus	152	17,298	Hexamer	1nue (+GDP)	Moréra et al. (1995)
Dd-NDPK	NDPK from *Dictyostelium*	(d)NDP	Cytosol	155	16,794	Hexamer	1lwx (+AZTDP), 1f3f (+d4TTP), 1mn9 (+ribavirinTP), 3fkb (+PMPADP)	Xu et al. (1997a), Meyer et al. (2000), Gallois-Montbrun et al. (2003) and Koch et al. (2009)
hPGK	Phosphoglycerate kinase EC 2.7.2.3	ADP, L-CDP	Cytosol	417	44,615	Monomer	2zgv (+ADP) 3c3c (+L-CDP)	Gondeau et al. (2008)
hCK-B	Creatine kinase B EC 2.7.3.2	ADP	Cytosol	381	42,644	Dimer	3b6r (+ADP)	Bong et al. (2008)
hPyr-Kin	Pyruvate kinase EC 2.7.1.40	ADP Fructose 1,6 diP	Cytosol	550	62,500	Trimer	1t5a (+F1,6dP)	Dombrauckas et al. (2005)

Properties of major human nucleoside diphosphate kinases (NDPK) and other kinases able to add a third phosphate to antiviral nucleoside analogs diphosphate. The data on cellular localization, molecular weight, number of amino-acid are from SwissProt (http://www.expasy.ch/sprot). PDB entries codes refer to X-ray structures deposited in the Protein data bank (PDB, http://www.rcsb.org/pdb). Dd-NDPK, a model for human NDPKs, has been studied in complex with a number of antiviral analogs.

Table 7
Catalytic properties of human enzymes catalyzing the last phosphate addition to some antiviral compounds.

Substrate	hNDPK-A k_{cat}/K_M (M^{-1} s^{-1})	hPGK k_{cat}/K_M (M^{-1} s^{-1})	Creatine K (human BB) k_{cat}/K_M (M^{-1} s^{-1})	Rabbit muscle Pyr Kin k_{cat}/K_M (M^{-1} s^{-1})
TDP	2×10^6 [a,b]			
AZTDP	2×10^2 [a,b,c]	+ [k]	nd [k]	nd [j]
D4TDP	2.6×10^3 [a,b]	+ [j, k]	+ [j,k]	++ [j,k]
CDP		250 [i]		
dCDP	2×10^5 [d]	<10 [i]		2×10^3 [ex from, f]
L-dCDP	nd	+		
L-3TCDP	Low	800 [i]		
Cidofovir-P	30 [ex from d,f]	–	Low [f (bovine heart)]	20 [ex from, f]
dGDP	7.4×10^6 [g]	9.5×10^5 [g]	3×10^4 [g]	2.7×10^4 [g]
dADP	3.6×10^5 [d,h]	5.2×10^4 [h]	1.3×10^6 [h]	9.3×10^4 [h]
PMPAP (tenofovir-P)	410 [h]	nd [h]	3.3×10^5 [h]	620 [h]

a = (Schneider et al., 2000), b = (Meyer et al., 2000), c = (Bourdais et al., 1996), d = (Schaertl et al., 1998), f = (Cihlar and Chen, 1996), g = (Feng et al., 2004), h = (Koch et al., 2009), i = (Gallois-Montbrun et al., 2004), j = (Krishnan et al., 2002a), and k = (Hsu et al., 2007).
The catalytic parameters were obtained at the steady state conditions in the presence of saturating ATP or at low substrate concentration, except in references a, b, d, which report data measured on NDPK under pre-steady-state conditions by monitoring the time-dependent change in intrinsic protein fluorescence, related to the phosphorylation state of the catalytic histidine (Schneider et al., 1998). The phosphodonors were 1,3-bisphosphoglycerate, creatine phosphate and phospho(enol)pyruvate in saturating amounts for respectively PGK, creatine kinase and pyruvate kinase.
"ex from" = extrapolated value from, – = no available data and + = activity not quantified.

the phosphate from the donor to the acceptor, the NDPK reaction implicates a covalent intermediate P–E, in which the enzyme is phosphorylated on a histidine. Thus, the transfer goes through two successive "ping-pong" steps (Garces and Cleland, 1969):

$$NTP + E \longleftrightarrow NDP + P\text{–}E$$

$$N'DP + P\text{–}E \longleftrightarrow N'TP + P\text{–}E$$

Each step is fully reversible, and the second is the first in reverse after the acceptor N′DP has replaced the NDP product. The phosphohistidine intermediate is stable for an hour or so in the absence of an acceptor substrate. This mechanism, now documented by a wealth of biochemical, kinetic and structural data (Janin and Deville-Bonne, 2002), is shared with a few other metabolic enzymes (Puttick et al., 2008). The reaction can be followed in either direction by classical spectrophotometric assays using coupled enzymes (Blondin et al., 1994) or by pre-steady-state fluorometric assays (Schneider et al., 1998; Schaertl et al., 1998). Table 7 reports kinetic data on the phosphorylation of the diphosphate derivative of the nucleotide analogs, and Table 8, data obtained with the triphosphate forms, which are the substrates of reverse transcriptase. The dNTP/dNDP equilibrium constant is essentially the same for the natural nucleotides and the analogs (Deville-Bonne et al., 1996).

Table 8
Catalytic parameters of the reverse reaction catalyzed by NDPK and PGK on the triphosphate form of natural nucleosides and some antiviral analogs.

k_{cat}/K_M (M^{-1} s^{-1}) substrate	hNDPK-A k_{cat}/K_M (M^{-1} s^{-1})	hPGK k_{cat}/K_M (M^{-1} s^{-1})
dTTP	1×10^6 [a,b]	110
AZTTP	75 [a,b,]	15 [c]
d4TTP	750 [a,b]	15 [c]
L-dTTP	1.7 [c]	500 [c]
dCTP	4.2×10^5 [d]	<10 [c]
L-dCTP	1 [c]	1.2×10^3 [c]
L-3TCTP	0.2 [e]	600 [c]
CidofovirPP	–	–
dGTP	4.7×10^6 [c,d]	1.3×10^5 [c,d]
AcyclovirTP	30 [d,f]	2.5×10^4 [d,f]

a = (Schneider et al., 2000), b = (Meyer et al., 2000), c = (Gallois-Montbrun et al., 2004), d = (Gallois-Montbrun et al., 2003), e = (Kreimeyer et al., 2001), and f = (Gallois-Montbrun et al., 2002).
NDPK activities were measured under pre-steady state conditions by fluorescence stopped-flow experiments. PGK activities were measured with 5 mM 3-phosphoglycerate and several concentrations of NTP donor.

NDPK was identified more than 50 years ago (Berg and Joklik, 1954), it is abundant in most living organisms and cell types, and its catalytic activity is high, with a turnover rate (k_{cat}) that often exceeds 1000 s^{-1} and k_{cat}/K_m values near the limit fixed by the substrate diffusion (Lascu and Gonin, 2000). Thus, the enzyme is commonly assumed to be the main source of the four dNTPs substrates of DNA polymerase, and of the CTP and UTP needed by RNA polymerase and many other metabolic enzymes. The biosynthesis of these compounds should be an essential function in all organisms, yet bacteria such as *Mycoplasma* lack the *ndk* gene that codes for NDPK (Bilitou et al., 2009), and its deletion in *E. coli* or *Saccharomyces cerevisiae* does not prevent growth, implying that other enzymes than NDPK can contribute to the production of the NTP's and dNTP's needed by the cell.

In mammals including human, NDPK is the product of the *nm23* (non-metastatic 23) family of genes, which comprises up to ten members. The genes are thus called because the first human gene, now called *nm23-h1*, was identified as a metastasis suppressor; its RNA levels were highest in cells and tumors of relatively low metastatic potential in two experimental systems, murine melanoma cell lines and rat mammary carcinoma (Steeg et al., 1988); *nm23-h1* was shown to be closely related to the *awd* development gene in *Drosophila* (Rosengard et al., 1989), and its product proved to be NDPK A, an isozyme present in erythrocytes and all other human cells (Gilles et al., 1991); *nm23-h2* codes for NDPK B, the other major isozyme; the rest of the family codes for minor or mitochondrial NDPK's, and for proteins with no established activity (Boissan et al., 2009). NDPK A and B have closely related sequences (88% identity) and very similar enzymatic properties (Table 7) (Lascu and Gonin, 2000), but they bear surface charges of opposite signs, and they perform a bevy of cellular functions not obviously related to nucleotide synthesis (Mehta and Orchad, 2009). The deletion of the single gene in *Drosophila*, or of the two major genes in mice, strongly affects development (Bilitou et al., 2009; Postel et al., 2009). Rather than to nucleotide metabolism, the deletion phenotypes may be related to the capacity of NDPK A and B to phosphorylate histidines on other proteins (Klumpp and Krieglstein, 2009), or to interact with DNA (Postel et al., 1993; Dexheimer et al., 2009) and with lipid vesicles (Tokarska-Schlattner et al., 2008; Baughman et al., 2008; Nallamothu et al., 2009). Recent data indicate that they also interact with proteins of adhesive intercellular junctional complexes such as plakoglobin, cadherins and alpha-catenin, resulting in increased expression and stability, which could provide a mechanism for the tumor/metastasis suppressor activity (Aktary et al., 2010).

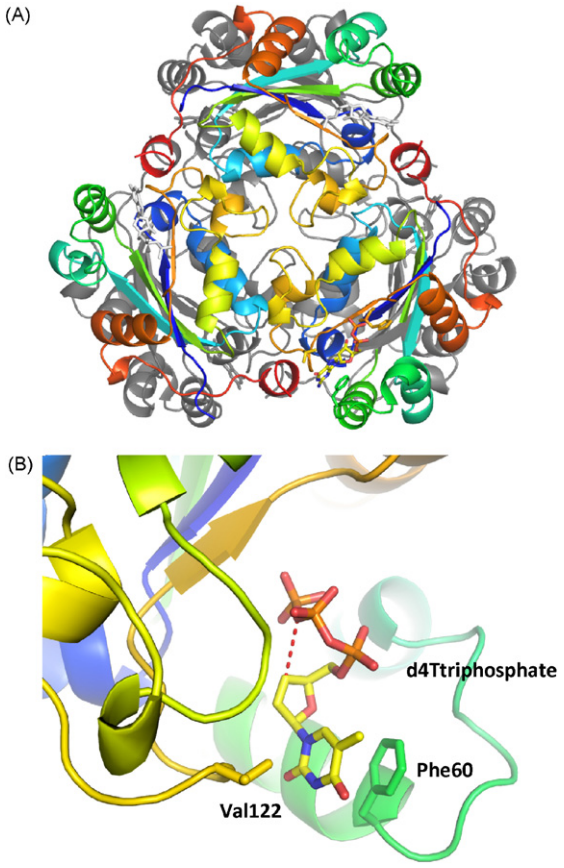

Fig. 11. Nucleoside diphosphate kinase. (A) The human NDPK B hexamer with bound GDP (PDB entry 1nue). (B) d4T triphosphate bound to the H122G mutant of *Dictyostelium* NDPK (PDB entry 1f3f). In the mutant, the active site histidine is replaced by a glycine to prevent the γ-phosphate transfer that would occur in the wild type enzyme. Note the stacking of the thymine base at the entrance of the active site and the C–H. . .O hydrogen bond (red dots) that links the C3′H group of the modified ribose to the oxygen bridging the β- and γ-phosphate. Albeit weaker than the 3′OH. . .O bond shown in Fig. 4 with a natural substrate, the C–H. . .O bond contributes to catalysis and makes d4TDP a better substrate of NDPK than a dideoxynucleotide.

NDPK A and NDPK B are homo-hexamers (Fig. 11A), a property they share with all other NDPK's except a few that are tetramers in bacteria. Their short (152 residues) polypeptide chains have the same fold as in other eukaryotic and prokaryotic NDPK's for which X-ray structures are available (Janin et al., 2000). The X-ray structures of complexes with natural substrates or analogs show that the reactive histidine (His118 in the two major human isoforms) is placed at the bottom of the binding pocket. The nucleotide enters the pocket with the phosphate moiety first, while the base fills a cleft near the protein surface (Fig. 11B). The aromatic cycle of the base packs against hydrophobic side chains, but its polar groups do not interact with the protein, except in guanine, the amino group of which H-bonds to the C-terminal glutamate of an adjacent subunit in the hexamer (Moréra et al., 1995). This peculiar mode of binding explains why most NDPK's including human NDPK A and NDPK B take all common nucleotides as substrates.

The nature of the base affects the catalytic efficiency, but the ratio between guanine, the best, and cytosine, the worse, is only 30 (Schaertl et al., 1998). The base-binding site also accommodates non-natural bases such as the triazole-carboxamide moiety of ribavirin, a ribonucleoside analog that is used in the therapy of chronic hepatitis C infection. Phosphorylated ribavirin derivatives are good substrates of NDPK A, which processes them at a rate comparable to cytosine-containing substrates (Gallois-Montbrun et al., 2003). Other analogs modified on the base but not the sugar, for instance BVdUDP, are probably also efficiently processed. In addition, the substrate promiscuity of NDPK is of great value to chemists, who commonly use it to prepare the triphosphate derivatives of all sorts of nucleoside analogs (Wu et al., 2004).

The nature of the sugar affects the enzymatic activity much more than the base, and this has profound consequences on viral therapy. The stereospecificity of hNDPK-A is limited to D-nucleotides, and the enzyme is inactive on L-compounds such as 3TC-DP, 3TC-TP, L-NTP and L-dNTP (Kreimeyer et al., 2001; Gallois-Montbrun et al., 2004). NDPK generally processes 2′-deoxynucleotides less efficiently than ribonucleotides, but by a factor of only 2–5. In contrast, its activity drops dramatically on analogs that lack a 3′-OH, and this results from the catalytic mechanism itself. When ATP binds to a kinase, for instance a protein kinase, the triphosphate moiety generally extends away from the sugar. In NDPK, it folds back towards the sugar, allowing a Mg++ ion to ligate all three phosphates, and the 3′-OH group of the sugar to donate a H-bond to the oxygen atom bridging the β- and γ-phosphates (Fig. 4) (Moréra et al., 1994; Chen et al., 2003). All other natural NDPK substrates bind in that way (Moréra et al., 1995; Janin et al., 2000). The bridging oxygen is the leaving group when the γ-phosphate is transferred to the active site histidine, and the attacking nucleophile in the reverse reaction. It acquires a negative charge in both the transition state and the product, and the H-bond from the 3′-OH stabilizes that charge, accelerating the transfer by at least four orders of magnitude (Schneider et al., 1998). Thus, catalysis is assisted by the substrate, the 3′-OH playing a part that is left to a protein group in most other kinases. The contribution of the H-bond is lost in substrates that lack a 3′-OH. As a result, the catalytic efficiency of NDPK A drops by a factor of 10^4 to 10^5 on AZTTP and d4TTP (Tables 7 and 8) (Schneider et al., 1998). Steady-state kinetics performed on the related *Dictyostelium* enzyme show that the loss in efficiency comprises both a drop in k_{cat} and a increase in K_M (Gonin et al., 1999). In AZT, the 3′-azido group that replaces the 3′-OH cannot give a H-bond, and it clashes with protein side chains in the binding site (Xu et al., 1997a). As a consequence, AZT triphosphate is about as poor a substrate of NDPK A as ddTTP. The d4T dehydro sugar ring has a double bond between C2′ and C3′. This increases the polarity of the C3′-H bond, and allows the formation of a C–H. . .O bond to the bridging oxygen, seen in the X-ray structure of the triphosphate derivative bound to *Dictyostelium* NDPK (Fig. 11B) (Meyer et al., 2000). Albeit weaker than a OH. . .O bond, the C–H. . .O makes d4T diphosphate a significantly better substrate than ddTDP (Schneider et al., 2000).

NDPK may also be responsible for the production of the active form of the acyclic nucleoside phosphonate drugs. The X-ray structure of tenofovir phosphate, an analog of dADP, and tenofovir diphosphate, an analog of dATP, bound to the *Dictyostelium* enzyme (Koch et al., 2009) shows that the analogs bind at the same site as natural substrates, but in a very different conformation. The adenine base is flipped upside down relative to its orientation in ATP; the methoxypropyl linker that replaces the sugar makes only non-polar interactions with the protein, and the phosphonate group is displaced from the position occupied by the α-phosphate of a normal substrate. Nevertheless, tenofovir phosphate is phosphorylated by NDPK, 10^3-fold more slowly than dADP, but still much faster than adenosine phosphonacetic acid, a related compound with a shorter linker between the adenine base and the phosphonate group, which binds NDPK in a conformation similar to that of a natural substrate, yet its phosphorylation is too slow for the turnover rate to be measured (Chen et al., 2005).

The pharmaco-modulation of nucleoside analogs, which aims to improve the affinity for their target, can affect their activation by kinases. The substitution of the α-phosphate oxygen of a dNTP by a borano group (BH_3^-) mimics the transition state of the nucleotide

incorporation by reverse transcriptase (Deval et al., 2005; Frangeul et al., 2007). The substitution restores the chain termination upon AZT and d4T incorporation that is lost in drug-resistant reverse transcriptase. It also makes the diphosphate derivatives of the analogs better substrates of hNDPK (Meyer et al., 2000), but results in the loss of phosphorylation by hAMPK (Topalis et al., 2008). In consequence, α-substituted derivatives such as the α-borano derivatives potentially active on resistant viruses may have to be formulated as "pronucleotides" already containing two phosphate groups in order to bypass the AMPK step (Wagner et al., 2000; Jessen et al., 2008).

5.2. Phosphoglycerate kinase and other human kinases

Among the enzymes that catalyze the phosphorylation of GDP and dGDP, several are active on acyclic analogs. In addition to nucleoside diphosphate kinase, they include pyruvate kinase, creatine kinase, phosphoglycerate kinase, succinyl-CoA synthetase, phospho enolpyruvate carboxykinase and adenylo-succinate synthetase (Miller and Miller, 1982). Cidofovir monophosphate is a poor substrate for pyruvate kinase, creatine kinase and NDPK (Cihlar and Chen, 1996) (Table 7).

The phosphorylation of antiviral nucleoside analogs by human phosphoglycerate kinase (hPGK) has been recently studied. This glycolysis enzyme catalyzes a reversible phosphate transfer between 1,3-biphosphoglycerate and ADP, generating 3-phosphoglycerate and ATP. Dr Y.C. Cheng (Yale university) was the first to show the phosphorylation of the diphosphate derivatives of thymidine analogs by hPGK (Krishnan et al., 2002a,b). We confirmed the result, and found that, although much less efficient than GTP as the phosphate donor in the reverse direction, AZT-TP and d4T-TP are comparable to dTTP (Gallois-Montbrun et al., 2004) (Table 8). Compared to dGTP acyclovir-TP is a good substrate for hPGK but not for hNDPK (Gallois-Montbrun et al., 2002, 2003). Moreover, hPGK lacks enantioselectivity. The X-ray structure of complexes with the L- and D-forms of ADP and CDP (Gondeau et al., 2008) shows the base to occupy a non-specific hydrophobic clamp with a water-filled cavity behind it that allows a high flexibility in the interaction (Gondeau et al., 2008). Given the broad substrate specificity of hPGK, this enzyme may be responsible for the cellular activation of many of the nucleoside analogs that NDPK A and B are unable to phosphorylate, and especially those in L-series.

6. Improving the kinases efficiency and changing their specificity

The thymidine kinase of HSV-1 can be used as suicide gene in the gene directed enzyme prodrug therapy (GDEPT) of cancer (Balzarini et al., 1985; Moolten and Wells, 1990; Kokoris et al., 2000; Culver et al., 1992; Portsmouth et al., 2007; Both, 2009). The viral protein is not toxic *per se*, but its expression in a tumor leads to the formation of a highly toxic metabolite, phosphorylated ganciclovir, when the much less toxic prodrug ganciclovir is applied. The method can in principle be applied to other enzymes and prodrugs, if the catalytic efficiency of the enzyme can be improved for the prodrug and/or decreased on natural substrates. Kinases of various origins including human, and other gene/prodrug combinations have been considered in prodrug therapy, aiming to suppress the immune response and allow tumoral delivery by oncolytic viruses (Thorne et al., 2005).

Two basic strategies are used to improve the efficiency and specificity of an enzyme: (i) "smart design", a rational structure-based approach based on site-directed mutagenesis and a 3D-structure and (ii) directed evolution, by generating molecular diversity and applying a convenient selection procedure, such as a selection based on better efficiency for antiviral nucleoside analogs reaction in cells transformed by the mutated enzymes (Hida et al., 2007).

Structure-based site-directed mutagenesis has proved to be an effective way to enhance enzyme specificity. Given a kinase that has a high activity on natural substrates and a low activity on the analogs, a few amino acid substitutions may generate an enzyme with a very different specificity (Knecht et al., 2002). Thus, human NDPK A has a very low efficiency on all nucleotide analogs that lack a 3′-OH group. This can be improved by two point mutations: N115S, which introduces the –OH group of a serine at the active site of the enzyme, and L55H, based on the sequence of the more efficient NDPK of *Dictyostelium discoideum*. The catalytic efficiency of the double mutant enzyme on the derivatives of AZT, d4T, ddT and acyclovir, is about 100 times that of wild type NDPK A (Gallois-Montbrun et al., 2002). Similarly, a human deoxycytidine kinase that bears the two mutations R104M-D133A has a substrate repertoire limited to pyrimidines and phosphorylates L-dT better than the D-enantiomer (Hazra et al., 2009).

Thymidylate kinase represents another remarkable example of how site-directed mutagenesis can be used to improve enzyme efficiency on prodrugs. Human and yeast TMPK phosphorylate AZT-MP 200 times slower than dTMP, but the ratio is only 2 with the *E. coli* enzyme (Lavie et al., 1998a,b). Based on the 3D-structure of the protein, the *E. coli* LID region was engineered into hTMPK, and an arginine residue in the P-loop replaced by a glycine. The mutant hTMPK has approximately 200 times more activity on AZT-MP than the wild-type, and it phosphorylates AZT-MP better than dTMP (Brundiers et al., 1999). In cells transduced with a lentiviral vector, the mutant hTMPK selectively induces apoptosis when AZT is added as a prodrug (Sato et al., 2007). Moreover, when this highly active hTMPK is expressed in T lymphocytes infected by AZT-resistant HIV, the intracellular concentrations of AZT metabolites increases to a level that overcomes the viral resistance to the drug (Lavie et al., 2008).

Directed evolution regroups a variety of techniques that allows proteins to evolve rapidly. Molecular biology tools such as random mutagenesis, cassette mutagenesis and gene recombination can generate billions of mutants in a single experiment that may yield, after efficient screening, improved enzymes for gene-directed enzyme prodrug therapy (Georgiou, 2000; Hida et al., 2007; Olsen et al., 2000). The reshuffling of HSV-1 and HSV-2 TK genes yielded a TK that has an enhanced ability to phosphorylate AZT, with a lower K_M for the analog and a decreased specificity for 2′-deoxythymidine (Christians et al., 1999). More recently, Lutz and co-workers have reported a method for improving kinase specificity for nucleoside analogues based on directed evolution. Their selection is made on a fluorescence-activated cell sorter (FACS) using fluorescent analogs that become trapped in the cells after phosphorylation. This approach was validated on the *D. melanogaster* deoxynucleoside kinase with 2′,3′-dideoxy-thymidine (ddT). The selection yielded a mutant enzyme with a 6-fold higher activity on ddT and a weak activity on 2'-deoxythymidine. The overall change in specificity was 10^4-fold (Gerth and Lutz, 2007; Liu et al., 2009).

The most studied enzyme for improving specificity is the viral HSV-1 TK. In contrast to the strict specificity of the endogenous hTK1, the viral enzyme phosphorylates a large variety of nucleoside analogues containing purines as well as pyrimidines (see paragraph 3). Several groups have attempted to optimize the phosphorylation of ganciclovir and acyclovir while decreasing that of 2′-deoxythymidine and thymidylate by either by random mutagenesis, rational design based on 3D structure, or by combining the two approaches. The exceptionally broad substrate specificity of the enzyme has been related to the amino acid triad His-58, Met-128 and Tyr-172 (Pilger et al., 1999). Residue Gln-125, which mediates the preferred binding of thymine base, was changed to Asn, Asp, or

Glu, resulting in enzymes with a slightly improved activity on ganciclovir (Hinds et al., 2000). Mutating two alanine residues, located close to the thymine base, to bulkier residues (Tyr, Phe and His), resulted in enzymes with an enhanced activity towards ganciclovir both *in vitro* and *in vivo* (Black et al., 1996; Degrève et al., 2000; Balzarini et al., 2002a, 2006; Kokoris and Black, 2002; Candice et al., 2008). A variant enzyme with five substitutions proved to be superior to wild type HSV-1 TK in a gene therapy approach of a xenograft tumor model in nude mice (Black et al., 2001). Whereas it remains to be shown that the designed variants can have clinical applications as super suicide genes *in vivo*, it is important to recognize that they contain multiple amino acid substitutions that would have been difficult to rationally design. Mutant HSV-1 TKs also hold promise in a wide variety of other applications for example as a reporter gene combined with [^{18}F]-fluoro-5-ethyl-1-β-D-arabinofuranosyluracil for imaging tumors by positron emission tomography (Dotti et al., 2009). Suicide gene therapy with HSV-1 TK and ganciclovir is in clinical phase III trials (Palmer et al., 2006; Both, 2009), but gene delivery systems will need an improved transfection efficiency and target specificity before the method becomes clinical routine.

The second enzyme in the ganciclovir activation pathway is guanylate kinase (Fig. 3), and the construction of a fusion protein encoding HSV-1 TK and the mouse enzyme (mGMPK), has achieved significant results in killing tumor cells (Ardiani et al., 2009). Human TK1 is also used as a molecular target for experimental boron neutron capture therapy of brain tumors (Barth et al., 2008). Other possible targets for enzyme/prodrug development concern the thymidylate kinases of vaccinia virus (Topalis et al., 2005; Caillat et al., 2008; Auvynet et al., 2009) and *Plasmodium falciparum* (Kandeel et al., 2009). These two enzymes display broad-spectrum substrate specificities, they accept bulky analogues such as 5-bromo-vinyl-dUMP (BVdUMP) or purine containing analogs, and could serve as basis for the selection of new potent enzyme/prodrug pairs for anticancer therapies. As vaccinia viruses are efficient vectors for gene therapy and as oncolytic viruses that specifically target tumors (Thorne et al., 2007), a combination of the two approaches could have synergistic effects.

7. Conclusion

When a viral DNA polymerase or reverse transcriptase incorporates a nucleoside analog, the chain termination that occurs ought to be an extremely powerful way of blocking virus infection. But the analogs are only prodrugs, and their conversion into an active form is often very inefficient, implying that high doses must be taken and favoring the selection of resistant mutants. We have described the enzymes involved, and detailed the features that make some of them rate limiting in the activation process. The cellular nucleoside kinases in charge of the first phosphorylation can be bypassed by using acyclic phosphonates, or when the virus itself codes for the activity. The second step is inefficient with thymidine analogs that rely on thymidylate kinase for their activation, but relatively efficient with guanosine analogs such as ganciclovir. The last step is so poorly catalyzed by nucleoside diphosphate kinase, that other kinases must be responsible for the production of the triphosphate derivatives. In this case, the culprit is the enzyme chemistry rather than its substrate specificity. The most probable activation pathway can be reconstituted from present tables; for example, the L-enantiomer of deoxycytidine is easily accommodated by hdCK, then hUMP-CMPK, then by hPGK. As the bottleneck step depends on the nature of the prodrug, different analogs require different strategies. Those that rely on enzyme engineering and gene therapy have achieved significant success *in vitro*, but they are still far from the clinical stage. Meanwhile, pharmaceutical chemists must continue developing new analogs, and in doing so, they must take into account the properties of the kinases that will activate them.

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